

## Analysis of the Thermostable Direct Hemolysin (*tdh*) Gene and the *tdh*-Related Hemolysin (*trh*) Genes in Urease-Positive Strains of *Vibrio parahaemolyticus* Isolated on the West Coast of the United States

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Urease-positive (Ure+) and urease-negative (Ure-) strains of *Vibrio parahaemolyticus* isolated from patients on the West Coast of the United States between 1979 and 1995 were analyzed for the thermostable direct hemolysin (*tdh*) gene and the *tdh*-related hemolysin (*trh*) genes (*trh1* and *trh2*). The DNA colony hybridization method with the polynucleotide probes was used to determine the distribution of the genes. Of 60 Ure+ strains, 59 strains (98%) had the *trh* (either *trh1* or *trh2*) gene and 54 strains (90%) carried the *tdh* gene. The absence of the *trh* gene or a related sequence in an exceptional Ure+ strain was confirmed by Southern blot analyses. The stronger correlation with the *trh* gene than with the *tdh* gene was mostly attributable to strains possessing only the *trh2* gene. Of 25 Ure- strains, 20 strains (80%) had the *tdh* gene but none had the *trh* gene. These results indicate a very strong correlation between the Ure+ phenotype and the *trh* gene and are consistent with those reported for strains isolated in Asia. The Ure+ strains carrying the *trh* genes were not restricted to a unique group of the strains. The O4:K12 strains carrying the *trh1* gene have predominantly been isolated since 1979. However, strains of various non-O4:K12 serovars carrying either the *trh1* or the *trh2* gene became predominant after 1992. In addition, analysis by the arbitrarily primed PCR method revealed two subgroups within the selected Ure+ O4:K12 strains. Hybridization tests with oligonucleotide probes demonstrated that the *trh1* sequences of the West Coast strains differ to some extent from those of Asian strains. Nevertheless, a PCR method previously established to detect both the *trh1* and the *trh2* genes in Asian strains could detect 98% of those genes in the West Coast strains.

*Vibrio parahaemolyticus* can cause gastroenteritis in humans through seafood consumption. The early epidemiological investigations revealed a very strong association between the Kanagawa phenomenon (KP) and gastroenteritis (14, 28). KP is a beta-type hemolysis on a special blood agar medium, Wagatsuma agar, induced by thermostable direct hemolysin (TDH) that is produced almost exclusively by clinical strains (34). Therefore, TDH has been considered a major virulence factor of *V. parahaemolyticus*. Investigations of patients with traveler's diarrhea originating from an outbreak of gastroenteritis in the Maldives in 1985 revealed that KP-negative strains of *V. parahaemolyticus* produce a TDH-related hemolysin (TRH) but not TDH (4, 5). TRH and TDH share a common epitope(s) (5). The similarity of the two hemolysins was also confirmed at the molecular genetic level; the *tdh* and *trh* genes encoding TDH and TRH, respectively, shared ca. 70% nucleotide sequence identity (11, 18, 22). Molecular epidemiological evidence supporting the association of the strains possessing the *tdh* or the *trh* gene with gastroenteritis was obtained (29).

Among the biochemical characteristics of *V. parahaemolyticus*, urease activity has usually been considered negative; less than 10% of the strains were positive for urease activity in most investigations reported between 1963 and 1974 (3, 27, 36, 37). Isolation of urease-positive (Ure+) strains from patients with

gastroenteritis in various parts of the world attracted attention and recently stimulated many publications (1, 2, 6, 10, 13, 23, 25). The KP-negative phenotype of the Ure+ strains was pointed out in some of the reports (2, 10, 13). Very recent reports on examinations of clinical strains isolated in Asian countries and Brazil indicated that the ratio of Ure+ strains among the clinical strains is gradually increasing and that a strong correlation exists between urease production and the presence of the *trh* gene rather than that of the *tdh* gene (12, 24, 26, 30–32). The correlation between urease production and possession of the *trh* gene suggests that urease production can be used as one of the virulence markers. The correlation was perfect in some of the studies (12, 24, 30, 32) but was not in other studies (26, 31). Unlike in the *tdh* genes, significant nucleotide sequence variation exists in the *trh* genes in different strains isolated in Asia, and the *trh* genes could be clustered into two subgroups, *trh1* and *trh2*, which share 84% sequence identity (11, 18). However, this has not been fully considered in examination of the *trh* gene in Ure+ strains so far. Ignorance of the sequence variation might affect the results of oligonucleotide-based gene detection, such as hybridization with an oligonucleotide probe and PCR.

Many of the *V. parahaemolyticus* strains isolated recently on the West Coast of the United States demonstrated urease activity (7). There are reports that suggested a possible association of the Ure+ phenotype and the presence of the *tdh* gene in the strains isolated in this region (1, 8, 9). However, the strains isolated in the United States have not been examined

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TABLE 1. O:K serovar and presence or absence of the *tdh*, *trh1*, and *trh2* genes in Ure+ strains of *V. parahaemolyticus* isolated from patients on the West Coast of the United States

Yr of isolation	No. of strains	O:K serovar <sup>a</sup>	Presence of the following gene <sup>b</sup> :			Strain selected or isolated from an exceptional source <sup>c</sup>
			<i>tdh</i>	<i>trh1</i>	<i>trh2</i>	
1979	1	4:12	+	+	—	6190
1982	1	4:12	+	+	—	7912
1983	1	4:12	+	+	—	
1984	1	4:12	+	+	—	1516
1987	1	4:12	+	+	—	3215
1988	6	4:12	+	+	—	
	1	1:UT	+	+	—	
	1	4:63	+	+	—	7093
	1	4:73	+	+	—	
1989	4	4:12	+	+	—	5791
1990	6	4:12	+	+	—	4500, 4735, 5435
	1	1:22	+	—	—	2448
	1	11:UT	+	+	—	
	1	4:12	—	+	—	
	1	4:63	+	+	—	
1991	4	4:12	+	+	—	0702, 4362, 4693
	2	1:56	+	+	—	3992, 4950
	1	1:9	+	+	—	4834
	1	1:UT	+	+	—	1437
	1	3:59	—	—	+	5992
1992	3	4:12	+	+	—	
1993	3	4:63	+	+	—	
	1	1:23	—	—	+	6288 (blood)
	1	5:UT	—	—	+	5463
	1	4:12	+	+	—	5807
1994	2	4:9	+	+	—	8738
	1	4:12	+	+	—	6978
1995	3	4:12	+	+	—	9796
	3	4:63	+	+	—	
	2	1:56	+	+	—	
	1	1:9	+	+	—	9638
	1	1:56	—	—	+	
	1	11:15	—	—	+	
Control	1	3:6	—	+	—	AQ4037
	1	4:37	—	—	+	AT4
	1	1:1	+	—	+	275
	1	1:41	+	+	—	250
	1	5:11	+	+	—	225

<sup>a</sup> UT, untypeable.<sup>b</sup> +, present; —, absent.<sup>c</sup> Source of isolation, if other than feces, is indicated in parentheses.

for the presence of the *trh* gene to our knowledge. The present study was undertaken to examine the distribution of the *trh* genes and its correlation with the Ure+ phenotype in the strains isolated from the patients on the West Coast since 1979. We used suitable methods to distinguish and characterize the *tdh*, *trh1*, and *trh2* genes in the test strains.

## MATERIALS AND METHODS

**Bacterial strains.** The following strains of *V. parahaemolyticus* isolated from patients and submitted to the Microbial Diseases Laboratory, California Department of Health Services, were examined (Tables 1 and 2): 5 strains representing the Ure+ strains isolated between 1979 and 1987 (1) and 55 Ure+ and 25 urease-negative (Ure-) strains isolated between 1988 and 1995. All isolates were identified as *V. parahaemolyticus* by the presence of standard cultural and biochemical characteristics (7). Also included in this study were control strains of *V. parahaemolyticus* isolated in Asian countries (Tables 1 and 2): WP1 (20); AQ4037 (22); AT4 (11); and 225, 250, and 275 (30). Unless otherwise specified in Tables 1 and 2, the strains listed in the tables were isolated from fecal samples. The urease activities of the test strains were examined by the urea agar method (Christensen's method). The NaCl concentration of the medium was increased to 1% (7).

**O:K serovar.** The *V. parahaemolyticus* strains were grown and the O:K serovars were determined as described previously (30).

**Polynucleotide and oligonucleotide probes.** The polynucleotide probe specific to the *tdh* gene was prepared with a 415-bp DNA fragment internal to the *tdh1* gene of strain WP1 (17, 20). The polynucleotide probe specific to the *trh1* gene was prepared with a 334-bp DNA fragment internal to the *trh1* gene of AQ4037 (29). The polynucleotide probe specific to the *trh2* gene was prepared with a 419-bp DNA fragment internal to the *trh2* gene of strain AT4 (11). The DNA fragments were labeled by the random priming method with <sup>32</sup>P-labeled dCTP (11). The oligonucleotide probes specific to the *trh1* and *trh2* genes are the A1 and A2 probes described previously (11). The sequences of the A1 and A2 probes are identical to those of the *trh1* gene of AQ4037 and the *trh2* gene of AT4, respectively, and both are 20-mer synthetic oligonucleotides. The oligonucleotides were labeled at their 5' ends with <sup>32</sup>P-labeled ATP (11).

**DNA colony hybridization test.** DNA colony blots were prepared as described previously (17). The DNA colony hybridization tests with polynucleotide probes were carried out under high-stringency conditions as described previously (17). The DNA colony hybridization tests with oligonucleotide probes were performed under high-stringency conditions specifically determined for each probe (11).

**Southern blot analysis.** Cellular DNA was extracted by the method described previously (21). Digestion of DNA with restriction enzymes and gel electrophoresis were carried out as described previously (19). The Southern blots were prepared as described previously (20). Hybridization with the polynucleotide probe was performed under high-stringency conditions (in solution containing 50% formamide) or under reduced-stringency conditions (in solution containing 35% formamide) as described previously (22).

TABLE 2. O:K serovar and presence or absence of the *tdh*, *trh1*, and *trh2* genes in Ure- strains of *V. parahaemolyticus* isolated from the patients on the West Coast of the United States

Yr of isolation	No. of strains	O:K serovar <sup>a</sup>	Presence of the following gene <sup>b</sup> :			Strain selected or isolated from an exceptional source <sup>c</sup>
			<i>tdh</i>	<i>trh1</i>	<i>trh2</i>	
1988	5	4:8	+	—	—	
	1	4:73	+	—	—	
1989	5	4:8	+	—	—	
	1	3:UT	—	—	—	5728 (wound), 7018
	1	4:34	—	—	—	890 (ear)
1990	4	4:8	+	—	—	1171
	1	3:UT	—	—	—	
	1	4:10	+	—	—	
	1	UT:UT	—	—	—	
1992	1	1:56	+	—	—	
1993	1	1:56	+	—	—	
1994	1	8:41	+	—	—	
1995	1	4:55	+	—	—	5781 (blood)
	1	8:44	—	—	—	
Control	1	4:12	+	—	—	WP1

<sup>a</sup> UT, untypeable.<sup>b</sup> +, present; —, absent.<sup>c</sup> Source of isolation, if other than feces, is indicated in parentheses.

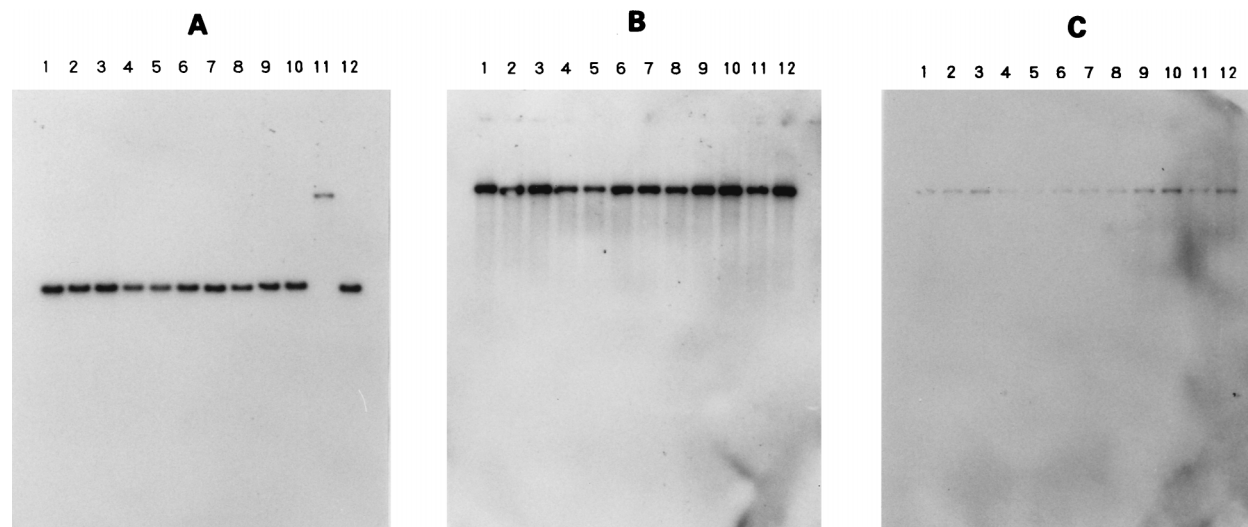


FIG. 1. Results of Southern blot hybridization analysis of the *Hind*III-digested cellular DNAs for selected strains of *V. parahaemolyticus* carrying both *tdh* and *trh*1 genes. The blots were examined by Southern blot hybridization analysis with the polynucleotide probes for the *tdh* (A), *trh*1 (B), and *trh*2 (C) genes under high-stringency conditions. The test strains (listed in Table 1) are 6190 (lane 1), 7912 (lane 2), 1516 (lane 3), 3215 (lane 4), 5791 (lane 5), 4693 (lane 6), 6978 (lane 7), 9796 (lane 8), 7093 (lane 9), 4950 (lane 10), 8738 (lane 11), and 9638 (lane 12). The sizes of the probe-positive bands were estimated by using bacteriophage  $\lambda$  DNA digested with *Hind*III as molecular size markers (data not shown): 2.8 kb (lanes 1 to 10 and 12) and 6.6 kb (lane 11) in panels A, 6.6 kb (all lanes) in panels B and C.

**PCR.** PCR for the detection of both *trh*1 and *trh*2 genes in the test organism was performed with the R2 and R6 primers described previously (33), except that *Taq* polymerase (Perkin-Elmer Cetus Corp., Norwalk, Conn.) was replaced with *TaKaRa Taq* (Takara Shuzo Co. Ltd., Otsu, Shiga, Japan) and that a Hybaid thermal reactor (model HB-TR1L; Hybaid Ltd., Middlesex, England) was used. A 5- $\mu$ l portion of the PCR-amplified mixture was resolved on a 5% polyacrylamide gel to detect 250-bp amplicons.

**AP-PCR.** Cellular DNA extracted as described above was used as the template for arbitrarily primed PCR (AP-PCR). PCR amplification was performed in a 30- $\mu$ l mixture composed of 0.2 mM (each) the four deoxynucleotide triphosphates, 25 ng of template DNA, 2.5 U of polymerase (*TaKaRa Ex Taq*; Takara Shuzo Co., Ltd.), 25 pmol of primer, and buffer ingredients. The deoxynucleotide triphosphates and buffer ingredients supplied by the manufacturer of the polymerase were used for the reaction. The oligonucleotide primers included in RAPD Analysis Primer Set (Pharmacia Biotech, Inc., Uppsala, Sweden) were evaluated in the preliminary experiment, and primer 1 (5'-d[GGTGC GGGA A]-3') and primer 2 (5'-d[GTTCGCTCC]-3'), which gave many sharp amplicon bands, were selected as the amplification primers. The reaction mixture was heated at 95°C for 4 min and was subjected to 45 cycles of PCR amplification in the Hybaid thermal reactor; 1 cycle consisted of denaturation at 95°C for 1 min, primer annealing at 36°C for 1 min, and extension at 72°C for 2 min. A 10- $\mu$ l portion of the PCR products was resolved on a 1.5% agarose gel.

**TDH detection.** The TDH produced by *V. parahaemolyticus* in the spent culture medium was detected by the immunological method based on the reversed-phase latex agglutination reaction with rabbit anti-TDH immunoglobulin G. The test strain was grown in a broth medium composed of 2% Bacto Peptone (Difco Laboratories, Detroit, Mich.), 0.5% D-mannitol, and 5% NaCl (pH 7.8), with or without shaking (180 rpm), at 37°C for 18 h. Appropriate dilutions of the culture supernatant were examined for the presence of TDH with a commercially available detection kit (KAP-RPLA; Denka Seiken Co., Ltd., Tokyo, Japan) according to the manufacturer's specifications. The level of TDH produced was expressed as the titer of the TDH that is the reciprocal of the highest dilution of the culture supernatant that gave a positive reaction.

## RESULTS

**Gene distribution and serovar.** The distribution of the *tdh*, *trh*1, and *trh*2 genes was determined by the DNA colony hybridization method with specific polynucleotide probes under high-stringency conditions. The O:K serovars of the strains were also determined. The results for Ure+ and Ure- strains are summarized in Tables 1 and 2, respectively. Five representative Ure+ strains of the O4:K12 serovar isolated between 1979 and 1987 (1) were shown to have not only the *tdh* gene but also the *trh*1 gene (Table 1). Of the Ure+ strains isolated

between 1988 and 1992, the O4:K12 strains carrying the *tdh* and *trh*1 genes were detected most frequently (Table 1). This type of strain also persisted after 1992, but the *tdh*- and *trh*1-bearing Ure+ strains of non-O4:K12 serovars became predominant during this period (Table 1). In addition, five Ure+ strains carrying only the *trh*2 gene have been isolated since 1991 (Table 1). To sum up, of the 60 Ure+ strains isolated on the West Coast of the United States since 1979, 59 strains had either the *trh*1 or the *trh*2 gene. This correlation of the Ure+ phenotype with the *trh* gene (98%) was stronger than that with the *tdh* gene (90%). The stronger correlation was mostly attributable to the strains possessing only the *trh*2 gene.

Compared with the Ure+ strains, Ure- strains were isolated less frequently after 1990 (Table 2). Of 25 Ure- strains isolated between 1988 and 1995, 20 strains (80%) had the *tdh* gene, but none had the *trh* gene. This and the results presented above indicate that the Ure+ phenotype is much more closely correlated with the presence of the *trh* gene than with that of the *tdh* gene.

**Persistence of Ure+ O4:K12 strains.** Eight representative O4:K12 strains, isolated between 1979 and 1995, that carried both *tdh* and *trh*1 genes and four *tdh*- and *trh*1-bearing strains of non-O4:K12 serovars were compared genetically to investigate whether a single clone of the O4:K12 serovar persisted on the West Coast. The polymorphisms of the length of the restriction fragments carrying the *tdh* and *trh* genes in the selected strains were first compared (Fig. 1). Previous studies demonstrated variations in the lengths of the *Hind*III fragments carrying the *tdh* and *trh* genes (11, 20). Therefore, *Hind*III-digested DNAs of the selected strains were compared by the Southern blot hybridization method. All selected strains with the exception of an O4:K9 strain (Fig. 1A, lane 11) had a 2.8-kb *tdh*-bearing *Hind*III fragment (Fig. 1A) and a 6.6-kb *trh*1-bearing *Hind*III fragment (Fig. 1B). Only the *trh*1-bearing fragments gave weak hybridization signals with the *trh*2-specific probe, confirming the absence of the *trh*2 gene (Fig. 1C). The results indicate that the nucleotide sequences around the *tdh* and *trh*1 genes in the selected strains are well conserved

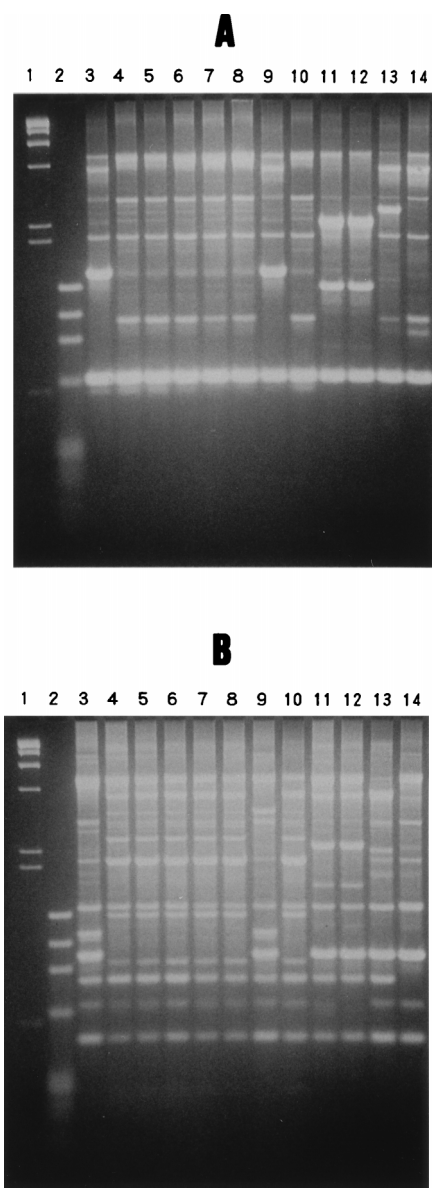


FIG. 2. Results of the AP-PCR assay for selected strains of *V. parahaemolyticus* carrying both *tdh* and *trh1* genes. The results obtained with primer 2 and primer 1 are presented in panels A and B, respectively. Lanes 1 and 2, molecular size markers: bacteriophage  $\lambda$  DNA digested with *Hind*III (lane 1) and  $\phi$ X174 DNA digested with *Hae*III (lane 2). Test strains (listed in Table 1) are 6190 (lane 3), 7912 (lane 4), 1516 (lane 5), 3215 (lane 6), 5791 (lane 7), 4693 (lane 8), 6978 (lane 9), 9796 (lane 10), 7093 (lane 11), 4950 (lane 12), 8738 (lane 13), and 9638 (lane 14).

regardless of the serovars and that a technique with higher resolution is needed to compare the selected strains.

Therefore, an AP-PCR method was used next. Two primers were used, and there was no essential difference in the results obtained with the two primers (primer 2 in Fig. 2A; primer 1 in Fig. 2B). On the whole, the AP-PCR patterns of the O4:K12 strains (lanes 3 to 10) differed significantly from those of the non-O4:K12 strains (lanes 11 to 14). Of eight selected O4:K12 strains, six strains isolated between 1982 and 1995 exhibited identical AP-PCR patterns (Fig. 2A and B, lanes 4 to 8 and 10). Two remaining O4:K12 strains isolated in 1979 and 1994 shared an identical AP-PCR pattern (Fig. 2A, lanes 3 and 9) or

had nearly identical AP-PCR patterns (Fig. 2B, lanes 3 and 9). The two subgroups within the O4:K12 groups distinguished by AP-PCR, however, shared several common amplicons. The results suggest that the O4:K12 strains carrying the *tdh* and *trh1* genes are fairly uniform but that there are at least two subgroups that have persisted over 13 or 14 years.

**Rare Ure+ strain lacking the *trh* gene.** Unlike other Ure+ strains, one Ure+ strain, strain 2448, lacked the *trh* gene and had only the *tdh* gene (Table 1). This was confirmed by Southern blot hybridization analysis under high-stringency conditions (Fig. 3A). The hybridization analysis was also performed under reduced-stringency conditions (Fig. 3B). Only the *tdh* sequences exhibited weak hybridization signals with the *trh1* and *trh2* probes, indicating that the *trh1*- or *trh2*-related sequence that is distinct from the *tdh* gene is also absent from strain 2448.

We were interested in comparing the level of TDH produced by strain 2448 with those produced by other Ure+ strains possessing the *trh* genes and by KP-positive strains (discussed below). The TDH level of strain 2448 was much higher than those of the other Ure+ strains carrying the *trh* gene but was not as high as those of KP-positive strains (strains WP1, 1171, and 7018; Table 3).

**Comparison of the gene detection methods.** We used the DNA colony hybridization method with specific polynucleotide probes under high-stringency conditions as the "gold standard" (Table 1). This is because it allows for as much as a 20% mismatch with the specific probes (15), and therefore, the target genes with significant sequence variation can still be detected. The hybridization signals of representative strains obtained with the *tdh*-, *trh1*-, and *trh2*-specific gene probes are presented in Fig. 4A to C. Significant strain-to-strain variation in the *trh1* and *trh2* gene sequences was documented previously (11). The two subgroups of the *trh* gene, however, could be distinguished by comparing the intensities of the hybridization signals obtained with the *trh1*- and *trh2*-specific gene probes since the two gene probes shared only 72% sequence identity (Fig. 4B and C).

The significant strain-to-strain sequence variation was reported previously in the variable regions of the *trh* gene, in which *trh1* and *trh2* sequences differed greatly (11). When a set of oligonucleotide probes was used to detect one such region of the *trh* genes (A1 and A2 probes specific to *trh1* and *trh2* sequences, respectively) in the DNA colony hybridization test under high-stringency conditions, 44% of the *trh1*-bearing strains and 95% of the *trh2*-bearing strains isolated in Asia gave positive results with the specific probes (11). The test strains isolated on the West Coast of the United States were examined by the same method in this study. The results obtained with the representative strains are shown in Fig. 4D and E. None of 53 *trh1*-bearing strains gave positive results with the A1 probe (compare Fig. 4B and D). Four (80%) of five *trh2*-bearing strains gave positive results with the A2 probe (compare Fig. 4C and E). The sequences of the variable region used for probe preparation were obtained from the Asian strains (11). The results therefore indicate that the *trh1* sequences of the West Coast strains differ to some extent from those of the Asian strains but that the *trh2* sequences are fairly conserved in both the West Coast and Asian strains.

We established previously a PCR method with oligonucleotide primers targeted to the conserved regions of the *trh* sequences so that all *trh1* and *trh2* genes present in Asian strains could be detected (33). We examined whether this PCR method can be used to detect the *trh1* and *trh2* genes carried by the West Coast strains. All 54 *trh1*-bearing strains and 4 of 5 *trh2*-bearing strains gave positive results. The 98% specificity

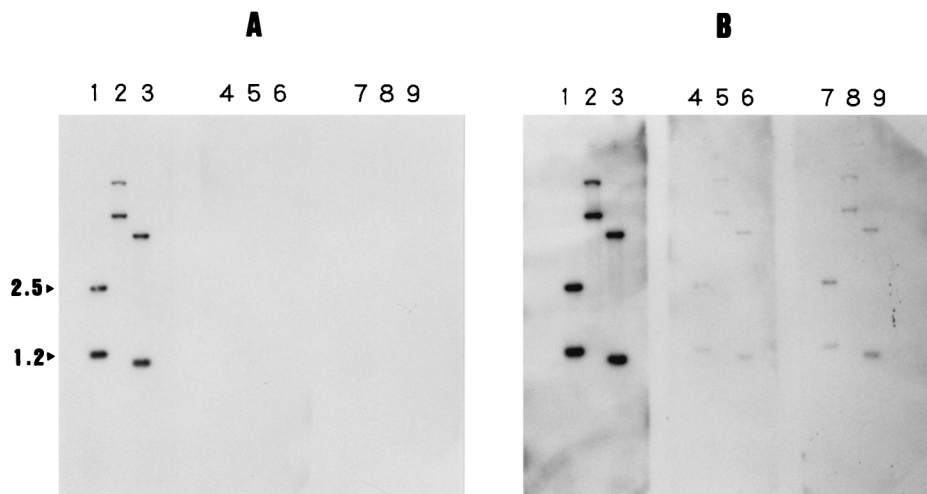


FIG. 3. Southern blot analysis of the DNA extracted from *V. parahaemolyticus* 2448. Lanes 1, 4, and 7, *Hind*III-digested DNA; lanes 2, 5, and 8, *Sal*I-digested DNA; lanes 3, 6, and 9, *Eco*RI-digested DNA. The blots onto which the DNAs in lanes 1 to 3, 4 to 6, and 7 to 9 were transferred were hybridized with the polynucleotide probes for the *tdh*, *trh1*, and *trh2* genes, respectively, under high-stringency (A) and low-stringency (B) conditions.

suggests that this PCR method is also applicable to the detection of the *trh* sequences in the strains isolated on the West Coast.

### DISCUSSION

It was demonstrated in this study that the *trh* genes are distributed in Ure<sup>+</sup> strains isolated from the clinical specimens on the West Coast of the United States. The Ure<sup>+</sup> phenotype was almost perfectly correlated with the possession of the *trh* (*trh1* or *trh2*) gene. This is consistent with the observation made with Asian strains. None of the five *trh2*-positive strains had either the *tdh* or the *trh1* gene (Table 1). Ninety-five percent of the *trh2*-bearing strains isolated in Asia had neither the *tdh* nor the *trh1* gene (11). Therefore, it seems to be a worldwide trend that the *trh2* gene usually does not coexist with the *tdh* and *trh1* genes.

The Ure<sup>+</sup> O4:K12 strains have frequently been isolated from both environmental and clinical sources on the West Coast (1, 8, 9, 23). The present study revealed that the Ure<sup>+</sup> O4:K12 strains carrying both the *tdh* and *trh1* genes have been persistently isolated since 1979 (Table 1). However, the AP-PCR patterns of the representative strains suggested that there are at least two subgroups which persisted over 13 or 14 years (Fig. 2). In addition, the strains of various non-O4:K12 serovars carrying either the *trh1* or the *trh2* gene became predominant among the Ure<sup>+</sup> strains isolated after 1992. These results do not agree very well with the supposition that the Ure<sup>+</sup> phenotype and the *trh* gene are stably maintained in a restricted group of the strains but rather suggest that the urease and the *trh* genes may be physically close and possibly present on a transferable genetic element. However, there was an exceptional Ure<sup>+</sup> strain lacking the *trh* or related gene. Evidence has been obtained that the *tdh* gene moved by an insertion sequence-mediated mechanism among the strains of *Vibrio* species in the past (35). It seems possible, therefore, that the *trh* gene may also be associated with the insertion sequence and that the gene may have been lost by an insertion sequence-mediated deletion mechanism in the exceptional Ure<sup>+</sup> strain. It would be interesting to examine whether the urease and *trh* genes coexist in a transferable genetic element such as a transposon flanked by insertion sequences.

KP-positive strains carry two copies of nonidentical *tdh* genes (*tdh1* and *tdh2*) but not the *trh* gene and produce TDH at high levels (11, 20, 21, 29). The strains carrying both *tdh* and *trh* genes produce TDH in much smaller amounts than those produced by KP-positive strains (29). It was also reported that Ure<sup>+</sup> strains carrying the *tdh* gene do not produce TDH or produce TDH at very low levels (24, 31). The rare Ure<sup>+</sup> strain, strain 2448, lacking the *trh* gene had *tdh* sequences on 1.2- and 2.5-kb *Hind*III fragments (Fig. 3A, lane 1). This and other hybridization patterns (Fig. 3A, lanes 2 and 3) were identical to those of KP-positive strains reported previously (16, 20). This suggested a possibility that strain 2448 has the *tdh* structure capable of producing high levels of TDH. The TDH level of strain 2448 was much higher than those of other Ure<sup>+</sup> strains carrying the *trh* gene but was not as high as those of KP-positive strains (Table 3). The result suggests a possibility that both urease production and the presence of the *trh* gene may contribute to low-level TDH production. Further studies are needed to examine this hypothesis. These include comparison of the promoter strengths between KP-positive strains and strain 2448 and investigations of the spe-

TABLE 3. Levels of TDH produced by selected strains of *V. parahaemolyticus*

Strain	Urease	Presence of the following gene:			TDH titer <sup>a</sup> of:	
		<i>tdh</i>	<i>trh1</i>	<i>trh2</i>	Stationary culture	Shaking culture
2448	+	+	—	—	<16	256
9796	+	+	+	—	NT	16
5807	+	+	+	—	NT	16
6190	+	+	+	—	NT	8
9638	+	+	+	—	NT	8
AQ4037	+	—	+	—	NT	<2
AT4	+	—	—	+	NT	<2
WP1	—	+	—	—	256	>512
1171	—	+	—	—	256	>512
7018	—	+	—	—	256	>512
890	—	—	—	—	NT	<2

<sup>a</sup> Reciprocal of the highest dilution of the culture supernatant that gave a positive reaction. NT, not tested.

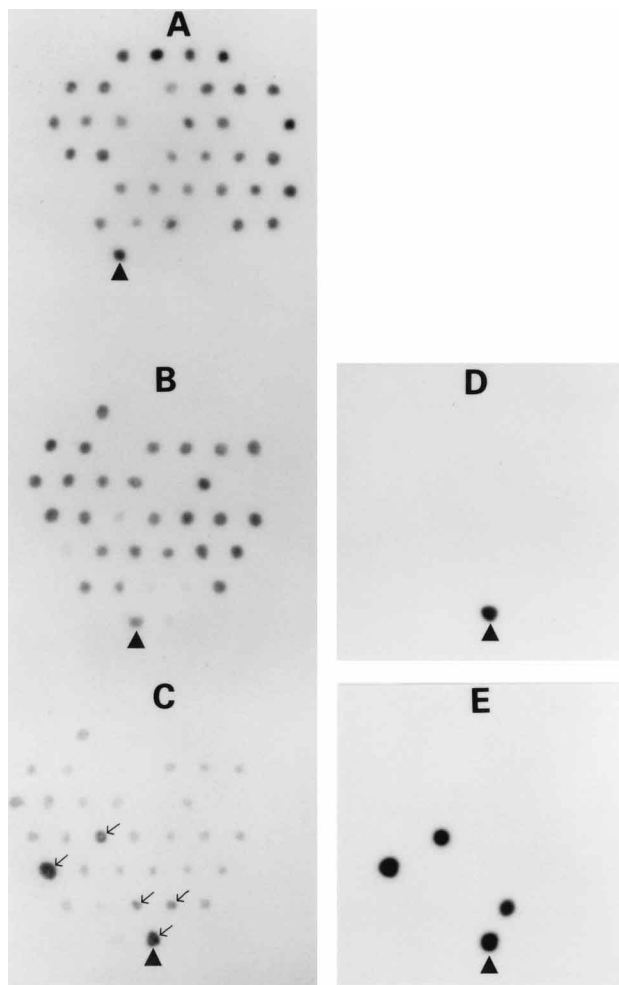


FIG. 4. Results of the DNA colony hybridization tests for representative strains. Forty test strains and three control strains were grown on identical spots on all the blots. The blots were hybridized with polynucleotide probes for the *tdh* (A), *trh1* (B), and *trh2* (C) genes and with oligonucleotide probes for the *trh1* (D) and *trh2* (E) genes under high-stringency conditions. The locations of the positive control strains for the respective probes are indicated by the filled triangles: WP1 (A), AQ4037 (B and D), and AT4 (C and E). The *trh2*-bearing strains are depicted by the arrows in panel C.

cific effects of urease and the *trh* gene on the transcriptional level of the *tdh* gene.

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